1991

IDENTIFICATION OF DQ ALPHA POLYMORPHISM USING THE POLYMERASE CHAIN REACTION

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December 18, 1991

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IDENTIFICATION OF DQ ALPHA POLYMORPHISM USING THE POLYMERASE CHAIN REACTION.

A thesis submitted for the degree Master of Science
at
Virginia Commonwealth University

by

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B.S. Rhode Island College, 1980

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Richmond, Virginia
December, 1991

DEDICATION AND ACKNOWLEDGEMENT

I would like to dedicate this thesis to my father, Richard Mello. His positive influence early in my life provides the support and encouragement in all my endeavors.

My deepest appreciation to my thesis advisor, Ronald Sauer, for his unending commitment toward achieving a high quality thesis. Special thanks to Teresa Nadder for her continual support and interest.

I would also like to thank all the people in the laboratory especially, Jean Mallory, Nellie Curry, Grace Mullinax, and Theresa Johnson. My sincere appreciation to Dr. Franklin Mullinax, for his invaluable support and for his decision to work with me from the very start.

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IDENTIFICATION OF DQ ALPHA POLYMORPHISM USING THE POLYMERASE CHAIN REACTION.

Abstract

Submitted in partial fulfillment of the requirements for the degree Master of Science.

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This was a study of detection systems for DQ alpha HLA polymorphism that could be exploited for the demonstration of simulated chimerism. Polymorphic segments of DQ alpha DNA were amplified by the polymerase chain reaction (PCR). Simulated chimerism was represented by a mixture of minor and major component DNA. The goal was to detect minor component DNA in the presence of major component DNA utilizing various laboratory techniques. Techniques studied included probe strip typing with the AmpliType HLA-DQ Alpha test kit, allele-specific amplification, polyacrylamide gel electrophoresis, restriction enzymes, and Southern transfer combined with a peroxidase detection system.

The AmpliType HLA-DQ Alpha test kit had a detection sensitivity of at least 0.2%. This is much better than the 10% detection sensitivity in non-PCR techniques. When the 3.0 DQ alpha type was mixed as the minor component with undiluted 1.1 DQ alpha type, the detection sensitivity for

the 3.0 DQ alpha type increased to a detection level of 0.1%.

The allele-specific primers were able to specifically amplify the minor component DNA in the presence of major component DNA. Major component DNA did not amplify and thus did not compete with the minor component DNA for Taq polymerase. The allele-specific primers provided an overall detection sensitivity of 0.2%.

Background interference prevented detection of minor component bands on both polyacrylamide gels stained with ethidium bromide and on Southern blots reacted with the peroxidase detection system.

CHAPTER I

Introduction

Organ transplantation is an important therapeutic tool. One example is bone marrow transplantation for treatment of hematological malignancies (Bortin & Rimm, 1989). Most human transplants are from allogeneic donors, that is, a human donor that is not genetically identical to the recipient.

The lack of an identical twin donor requires that bone marrow be carefully matched with the recipient (Sparkes, Crist, Gale, & Feig, 1977). When an appropriate donor is found, the host's malignant bone marrow is treated to eradicate all bone marrow cells. The transplant will infuse donor cells, and they will repopulate the bone marrow and remain as the only viable bone marrow cells.

If host bone marrow cells are not completely eradicated, two genetically different cell populations remain in the bone marrow. The presence of these two different cell populations in one host is termed chimerism.

A condition known as graft-versus-host disease (GVHD) may occur. GVHD is a condition caused by allogeneic donor lymphocytes reacting against host tissue. That is, donor T lymphocytes recognize host cells as

foreign and possibly activate host B lymphocytes to initiate an autoimmune response (Lindholm, Rydberg, & Stannegard, 1973). Death can result from bone marrow aplasia, liver failure, massive enteritis, progressive immunodeficiency, or secondary infection (Hansen, Woodruff, & Good, 1981). Chimerism detection is therefore an important procedure. No method has been sufficiently sensitive to detect a small number (e.g., less than 10%) of surviving residual host chimeric cells. The polymerase chain reaction (PCR) may overcome this obstacle.

The polymerase chain reaction is a relatively new procedure to amplify, in the test tube, a specific gene sequence into millions of copies in a simple, quick and automated reaction (Mullis, Faloona, Scharf, Saiki, Horn, & Erlich, 1986). This ability to amplify a specific deoxyribonucleic acid (DNA) fragment has had a tremendous impact on molecular biology research (Rodu, 1990) and forensic science (Jeffreys, Wilson, Neumann, & Keyte, 1988).

PCR technology has facilitated forensic tissue analysis (Jeffreys, Wilson, Neumann, & Keyte, 1988). This technique amplifies hypervariable regions of DNA, including repeating nucleotide sequences known as variable number of tandem repeats (VNTR), portions of the Y chromosome, and HLA-DQ alpha segments.

This application of PCR technology increased the sensitivity for the detection of chimeric cells, but its use was limited. The amplification of a portion of the Y chromosome from the DNA of male cells limited the application to male patients who received female bone marrow (Lawler,

McCann, Conneally, & Humphries ,1989).

This limitation was overcome by amplifying hypervariable DNA segments found in both males and females. The VNTR DNA segments (Chalmers, Sproul, Mills, Gibson, & Burnett, 1990) and the human leukocyte antigen (HLA) genes such as the DQ alpha gene (Gyllensten & Erlich, 1988) are such hypervariable DNA segments. Techniques for detecting the amplified DQ alpha gene are the subject of this study.

The HLA-D or major histocompatibility complex (MHC) class II genes are located on chromosome 6. The HLA-D genes are organized into three loci: HLA-DR, HLA-DQ, and HLA-DP. Each locus encodes for an alpha and a beta glycoprotein. It is the association of the alpha and beta glycoproteins that forms a heterodimer protein expressed on the surface of B lymphocytes, macrophages, and activated lymphocytes (Trowsdale, Young, Kelly, Austin, Carson, Meunier, Erlich, Speilman, & Bodmer, 1985).

Variation in the DNA base sequence, or polymorphism, found in MHC class II genes is seen near the amino terminal outer domain encoded by the second exon (Trowsdale, Young, Kelly, Austin, Carson, Meanier, So, Erlich, Speilman, & Bodmer, 1985). A 242 base pair fragment containing the hypervariable region encoding for a portion of the alpha chain of the HLA-DQ region (DQ alpha) has been amplified by the polymerase chain reaction. It is in this area that allelic variation occurs (Gyllensten & Erlich, 1988).

There are 8 known alleles of HLA-DQ alpha. There are four major alleles; A1, A2, A3, and A4. The types A1 and A4 can be subtyped into A1.1, A1.2, A1.3, and A4.1, A4.2, and A4.3, respectively (Cetus Corporation,

1990). The 8 DQ alpha types represent alternative forms of the DQ alpha gene and thus each is referred to as an allele. Two DQ alpha genes are inherited, one from each parent. If the two inherited DQ alpha genes are different, the patient is heterozygous for the DQ alpha gene; and the patient's tissue will type as two different DQ alpha types. If both inherited DQ alpha genes are identical, the patient will be homozygous for the DQ alpha gene; and the patient's tissue will type as one DQ alpha type (Gyllensten & Erlich, 1988).

The DQ alpha types can be detected and typed with the AmpliType HLA-DQ Alpha test kit from Cetus Corporation. This is the first commercially available test kit employing PCR methodology. The kit has primarily been used in forensic studies to type for DQ alpha genes using very small quantities of tissue (Cetus Corporation, 1990). The kit was used in the present study to detect known DQ alpha types in laboratory-prepared mixtures of DNA that simulate chimerism. Typing with the AmpliType kit is compared with alternative techniques involving restriction endonucleases, electrophoresis, allele-specific amplification, and Southern blotting with a peroxidase detection system.

Rationale for this Study

Chimerism following bone marrow transplantation may be involved in GVHD (Borgaonkar, Bias, Sroka, Hutchinson, & Santos, 1974). Therefore, detection of chimerism may be important. A technique such as PCR could help detect chimerism. This project focuses on the identification of DQ alpha polymorphism to detect DQ alpha types in laboratory-prepared

mixtures of DNA.

The rationale for examining DQ alpha type is the recognition that (1) the DQ alpha gene can be used as a marker to differentiate between individuals with different DQ alpha types, (2) chimeric individuals may have distinguishing DQ alpha types, and (3) published reports indicate PCR is effective in detecting chimerism (Lawler, McCann, Conneally, & Humphries, 1989; Chalmers, Sproul, Mills, Gibson, & Burnett, 1990). The rationale for using the DQ alpha test kit for detecting chimerism is that it combines the amplifying power of PCR with the ability to differentiate between the different DQ alpha types. (Cetus Corporation, 1990).

Statement of Hypothesis

The DQ alpha AmpliType test kit will not detect chimeric mixtures of major and minor DQ alpha DNA types and must be supplemented with, or replaced by alternative DQ alpha typing techniques.

Laboratory experiments include:

1) Evaluation of the AmpliType HLA-DQ Alpha test kit for sensitivity and specificity using DNA specimens from individuals of known DQ alpha types. Specimens include those tested singly and as mixtures from two individuals.

2) Kit analysis supplemented with allele-specific amplification. Allele-specific amplification may allow relatively small amounts of DNA of one DQ alpha type, or minor component DNA, to be amplified with greater efficiency in the presence of a larger amount of DNA, or major component DNA. The amplified DQ alpha DNA is detected by a combination

of gel electrophoresis and kit probe strip typing.

3) Restriction endonuclease enzymes used to cut amplified DQ alpha DNA at specific base sequence sites prior to examination by polyacrylamide gel electrophoresis. Since the DNA from the various DQ alpha types have different base sequences, one DQ alpha type can be cut while another DQ alpha type remains uncut. When examined by gel electrophoresis, the cut DQ alpha type reveals more than one band, while the uncut DQ alpha type is seen as one single band of 242 base pairs. This allows differentiation between DQ alpha types.

4) Detection sensitivity of the polyacrylamide gel will be investigated by using International Biotechnologies, Incorporated, Enzygraphic Web (Eastman Kodak Company Package Insert, 1991). Amplified DNA is electrophoresed in polyacrylamide gel and the gel will undergo Southern blotting onto a nylon membrane. The membrane is allowed to react with the Enzygraphic Web. The Enzygraphic Web is a support polymer coated with a detection system for peroxidase enzyme activity.

The results of these studies are presented in the following chapters. Chapter II discusses blood transfusions and isoantigens, MHC, HLA, and DQ alpha, HLA typing techniques, organ transplants, bone marrow transplantation, immunological tolerance and chimerism, polymerase chain reaction, Taq polymerase, streptavidin-biotin detection system, restriction endonucleases, and polyacrylamide gel. Methods, materials, and experimental design are included in Chapter III. The results are

contained in Chapter IV and the discussion is included in Chapter V.

CHAPTER II

Literature Review

Blood Transfusions and Isoantigens

In 1875, Landois discussed the adverse effects of transfusing blood between species. Blood transfusion between the same species was preferable; however, complications were common even between the same species (Landois, 1875).

Landsteiner's work in 1901 resulted in the discovery of the first human blood group system, the ABO system. Landsteiner mixed various different human blood serum specimens with washed erythrocytes from other donors and frequently observed agglutination. The serum factor that caused agglutination of blood from the same species was named isoantibody or isohemagglutinin (Landsteiner, 1931). The discovery of the ABO system was important because the transfusion of ABO incompatible red blood cells may lead to death.

Unexpected transfusion reactions continued despite ABO compatibility testing. The discovery of the second blood group system, the Rh system, by Landsteiner and Wiener in 1940 was significant for reducing

the number of hemolytic transfusion reactions and for defining hemolytic disease of the newborn. When rhesus monkey red blood cells were inoculated into guinea pigs and rabbits, the animals developed antibodies that caused agglutination of both rhesus monkey erythrocytes and the erythrocytes of about 85% of the human donors. This newly identified antigen was designated Rh, for the source of discovery, the rhesus monkey (Landsteiner & Weiner, 1940).

Antibodies to Rh antigens are responsible for a variety of in vivo hemolytic antigen-antibody reactions. For example, hemolytic disease of the newborn or erythroblastosis fetalis can be caused by maternal antibodies directed against in utero fetal Rh red cell antigens. The mother is exposed to foreign Rh antigen due to feto-maternal hemorrhage during delivery. This exposure may result in sensitization (antibody production). In subsequent pregnancies, the antibody may cross the placenta, attach to fetal Rh antigen, and the ensuing antigen-antibody reaction results in hemolysis and marked infant damage (Freda, Gorman, Pollack, & Bowie 1975).

Subsequent work revealed a variety of other isoantigens on the red blood cells. Some are sufficiently potent immunogens to cause transfusion reactions. Other isoantigens are primarily used to solve medicolegal and anthropological problems, but have yet to show any definite contribution to disease (Issitt & Crookston, 1984).

Isoantigens have also been identified on blood leukocytes and platelets. These markers are collectively designated human leukocyte

antigens (HLA) and are found on platelets, all white blood cells, and nucleated fixed tissues of the body (Dausset & Brecy,1954; Van Camp, Cole, & Petermans, 1977; Gill, 1978).

Major Histocompatibility Complex (MHC), HLA, and DQ Alpha

The major histocompatibility complex (MHC) is the HLA gene cluster located on the human chromosome 6 (Dausset, et.al., 1954; Van Camp, et.al., 1977; Gill, Cramer, & Runz 1978). The HLA gene cluster is divided into three distinct regions, based on the structure and function of the encoded protein product. The three protein products are MHC class I proteins, MHC class II proteins, and complement components.

MHC class I proteins consist of two polypeptides. The larger is encoded by the MHC in the HLA-A, B and C subregion and is noncovalently associated with the smaller peptide, β2 microglobulin. The smaller peptide is encoded outside the MHC region. These proteins are inserted into the cellular membranes of all nucleated cells and platelets where they serve as recognition molecules for identification of self by cytotoxic T lymphocytes.

The HLA-D subregion of the MHC encodes the class II proteins. These proteins are involved in the cooperation, interaction, and regulation between cells of the immune system. They consist of two noncovalently associated peptides, the alpha and beta chains; both are encoded by the MHC complex. The HLA-D subregion consists of three distinct loci: HLA-DQ, HLA-DR, and HLA-DP. These proteins are inserted into the cellular membranes of B lymphocytes, macrophages, and activated T lymphocytes.

The third protein class encoded by the MHC region are proteins of the classical and alternate complement pathways (Owen & Crumpton, 1980).

The HLA system is extremely polymorphic; that is, for each known locus, there are multiple potential alleles. For example, there are at least 23 distinct alleles at the HLA-A locus and at least 47 at the HLA-B locus (Zinkernagel, 1979).

Erlich utilized HLA-D polymorphism to examine small HLA-DQ alpha gene differences between two different cell populations (Erlich & Bugawan, 1989). The HLA-DQ alpha gene has four major allelic types. They are A1, A2, A3 and A4. Type A1 can be subtyped into A1.1, A1.2, A1.3. Type 4 can be subtyped into A4.1, A4.2 and A4.3 (Cetus Corporation, 1990). These hypervariable regions of polymorphism may be amplified by the polymerase chain reaction (PCR) in order to determine the HLA-DQ alpha type of a cell population.

HLA Typing Techniques

HLA-A, B and C loci (MHC class I) were originally typed by agglutination techniques, employing mixed leukocytes as target cells. The complement fixation test with a platelet target was an alternate procedure (Carpenter, 1977). Both techniques have been replaced by the microlymphocytotoxicity technique.

The microlymphocytotoxicity test employs antibodies specific for either HLA-A, B or C in the presence of rabbit serum complement. Specific antibody will react only with a complementary HLA type. Each HLA type is identified when it's specific antibody binds the HLA antigen, activating

complement. Complement activation causes the cells to be injured. Cells injured by an antibody-complement-antigen reaction allow a dye to penetrate. The cells become colored and are counted (Sanfilippo, Vaucha, Spees, Hilse, & Letor, 1984).

MHC class II typing is performed for the HLA-D region, specifically the HLA-DR loci. Mixed lymphocyte reactions are used to determine HLA-DR compatability between donor and recipient lymphocytes (Van Leverwen, Schmit, & Van Rood, 1973).

Organ Transplants

Blood transfusion and organ transplantation were very unsuccessful prior to accurate cell and tissue typing. The ABO and Rh identification largely solved the blood transfusion problem, but organ transplantation required matching additional cell factors such as the HLA gene products. Little suggested, even before the ABO and Rh systems were perfected, that several common, independently segregating, dominant genes in both donor and recipient, were crucial to allograft tissue transplants (Little, 1914). This prediction was proven true utilizing syngeneic mice, that is, mice inbred to become genetically identical. Tissue grafts within an inbred strain survived; whereas, grafts between two different strains did not survive. This demonstrated a vital genetic control of graft rejection or survival (Little, 1941).

Gorer demonstrated that genetic control of graft survival has an immunological basis and that tissues contain genetically determined markers (Gorer, 1937). In other words, tissues from a given individual

express markers that may be recognized as foreign by another individual of the same species. These alloantigens (present on the grafted tissue but absent in the recipient) are capable of eliciting an immune response and may destroy grafted tissue (Gorer, 1938). These observations were confirmed by Medawar using rabbit skin allografts (Medawar, 1944).

Multiply-transfused patients and multiparous females were observed to possess antibodies capable of agglutinating leukocytes. It was first proposed (Medawar, 1958), and subsequently shown (Gotze, 1977), that HLA incompatibility is the principle barrier to successful allografting. Both MHC class I and II antigens are significant cell surface proteins. They are the surface antigens that enable an individual to recognize a cell as self, while recognizing foreign cells as nonself. When foreign MHC class II antigens are recognized as foreign by host cells, the graft will be rejected.

The transplanted tissue type helps determine allograft transplantation success rate (Calne, 1973). The most frequently successful organ transplants in decreasing order of success are liver, kidney, heart, islets of Langerhans (pancreas), skin and bone marrow.

The first successful renal transplantation was performed in 1954 at the Peter Brent Brigham Hospital. Renal transplant is currently an accepted therapy for end-stage renal failure (French and Batchelor, 1969). Survival rate of renal transplants between HLA identical siblings is about 85% after five years. There is a 10-15% survival reduction for each donor and host incompatibility (Kissmeyer, 1974).

The first human liver transplantation was performed in 1963. Liver transplantation has limited application for end-stage liver disease treatment but may be a life-saving procedure. Liver matching is based on liver size and ABO compatability. HLA matching is not performed. There have been few reported hyperacute rejections due to HLA incompatabilities, but the basis of resistance to liver rejection is unknown (Starzl, Koep, Halgrimson, Hood, Chroter, Porter, & Weil, 1978). There is a 70-75% survival rate beyond one year (Starzl, Koep, Weil, Halgrimson, & Franks, 1979).

The first successful human heart transplantation was performed in 1967 by Barnard. Currently, the indication for cardiac transplantation is end-stage cardiac impairment due to coronary artery disease, cardiomyopathy, rheumatic heart disease, congenital heart disease, or benign cardiac tumors (Griep, 1979). Pretransplant immunologic evaluation consists of ABO blood typing, HLA typing, and screening for preformed anti-HLA antibodies. Unlike kidney transplantation, the opportunity to choose from several cardiac donors is rare.

To be acceptable for heart transplantation, only HLA crossmatch negative recipients are chosen. Donors bearing specific HLA antigens to corresponding recipient antibodies are avoided. Rejection is minimized with administration of corticosteroids, cyclosporine, and antilymphocyte globulin. Graft survival after one year is greater than 60% (Jamieson, Stinson, & Shumway, 1979).

The first successful pancreas transplantation occurred in 1966

(Lilleher, Simmons, Najarian, Weil, Uchida, Ruiz, Killstrand, & Goetz, 1970). Pancreatic transplants are not a life saving procedure but are a life enhancing procedure. Pancreas transplantation may correct carbohydrate metabolism abnormalities (Lee, Mauer, Brown, Sutherland, Michael, & Najarian, 1974). Both ABO and HLA compatability are necessary for a successful transplant. The present successful graft survival rate for one year is 40% and the patient survival rate is 70% (Sutherland & Kendall, 1985).

Bone Marrow Transplantation

Bone marrow transplantation is treatment for some advanced leukemia's, aplastic anemia, severe combined immunodeficiency disease (SCID) and a wide range of other malignant and non-malignant hematological diseases (Thomas, 1975).

ABO and HLA compatibility is desired. ABO incompatibility does not interfere with successful engraftment of stem cells but increases the likeliness of a hemolytic reaction. Marrow transplantation can be successful when ABO incompatability exists if plasma exchange is added to prevent hemolysis (Buckner, Clift, Sanders, Williams, Gray, Storb, & Thomas, 1978).

Bone marrow transplantation involves the transfer of multipotential hematopoietic stem cells to an irradiated and chemically-treated host bone marrow (Thomas, 1975). The transplanted bone marrow contains fully differentiated donor cells, which, when infused to the recipient bone marrow, repopulate the marrow.

If genetic differences are detected by donor cells, a graft- versus-host disease (GVHD) may follow. Recipient stem cells, skin epithelial cells, mucous membranes, gastrointestinal tract and liver are specially susceptible to GVHD. The severity of GVHD correlates to the degree of alloantigenic difference between donor and recipient. Death may result from marrow aplasia, liver failure, massive enteritis, progressive immunodeficiency or secondary infection (Hansen, Woodruf, & Good, 1981).

There is a three-step regimen to prevent GVHD. The patient first receives chemotherapy and total-body irradiation to destroy diseased tissue and establish immunosuppression (Thomas, 1975; Storb, Thomas, Buchner, Appelbaum, Clift, Deig, Doney, Hansen, Prentice, Sanders, Stewart, Sullivan, & Witherspoon, 1984; Thomas, 1983). Secondly, the donor and recipient are matched for ABO and HLA antigens. Finally, immunosuppression is continued post-transplantation; and the patient is carefully monitored for infection until mature donor neutrophils and lymphocytes are exported from the bone marrow (Kay, Powles, Sloane, & Farthing, 1980).

The ideal transplantation is between identical twins, to eliminate the risk of alloimmune mediated rejection or GVHD (Thomas, et.al., 1971). Identical twins are uncommon; the next best donor choice is an HLA-identical sibling (Parkman, Rosen, Rappeport, Camitta, Levig, & Nathan, 1976; Mickelson, Clift, Fefer, Storb, Thomas, Warren, & Hansen, 1981).

It is most desirable to have donor and recipient identical, but there

have been successful transplants between partially-matched family donor and recipient bone marrow. Delayed engraftment, complete rejection or GVHD occurs earlier and with greater frequency in partially-matched family transplants, but there is no significant reduction in survival rates (Clift, Hansen, and Thomas, 1978; Dupont, O'Reilly, Pollack, and Good, 1980; Beatty, Clift, Mickelson, Nisperos, Flournoy, Martin, Sanders, Stewart, Buckner, Storb, Thomas, & Hansen.1985).

Most patients (60%) lack both an identical sibling and partially-related donor. These patients are transplanted with unrelated, HLA donors (Hansen, Clift, Thomas, Buckner, Storb, & Gilbert 1980). These donors do not have identical loci within the HLA gene region on chromosome 6. They may have minor histoincompatabilities outside the HLA region. The number of transplantations with unrelated donors is too few to allow conclusions regarding their success rate.

Successful bone marrow transplantation varies greatly and depends on many variables. Animal experiments have clearly demonstrated the MHC to be the major factor causing graft rejection and GVHD (Eichwald, Hart & Eichwald, 1969; Klein & Park, 1973; Hood, Steinmotz & Malisson, 1983). Fatal GVHD can be avoided in mice, if donor and recipient are H-2 compatible (the equivalent of human MHC compatible). The H-2 complex contains a subregion, the I region, where the immune response is regulated. The I region produces "self" or Ia antigens. These Ia antigens, similar to human MHC class II antigens, were shown to govern lymphocyte interactions. Successful grafts following Ia matching resulted in reduced

GVHD in mice (Rodey, 1974). GVHD still occurs, despite H-2 matching and may be attributable to non-MHC or minor histocompatability antigens (Cantrell & Heldmann, 1972).

Immunological Tolerance and Chimerism

Long term survival of transplanted tissue requires engrafted tissue to be accepted as nonimmunogenic. This is especially true of bone marrow transplantation. Thus, a perfect match would allow each cell type to tolerate the other foreign cell type. This state of nonresponsiveness to foreign tissue is termed tolerance. Tolerance may be induced in both cellular and humoral immune systems and may be congenital or can be induced through a variety of immunosuppressive treatments (Dresser & Mitchinson,1968; Humphrey, 1976).

Tolerance was first recognized by Erlich while working with self antigens. It was learned that tolerance to non-self antigens can be induced in animals (Danforth & Foster, 1929). A major breakthrough in understanding tolerance mechanisms occurred in 1945. Normally, nontolerant grafts between non-identical cattle would be rejected, but nontolerant cattle became tolerant to each other if they exchanged embryonic blood following placental fusion. These non-identical cattle harbored two different cell types, yet were tolerant of both.

This phenomenon, in which two genetically different tissue types co-inhabit the same host is called stable chimerism (Billingham, Brent & Medawar, 1956). The recipient tolerated the donor cells, despite genetic differences that would normally cause an immune reaction.

Lack of tolerance between chimeric cells may result in chronic GVHD in bone marrow transplantation patients. Chimerism detection, is therefore an important procedure. HLA typing is a method used to monitor the chimeric status of bone marrow transplantation patients (Blazer, Soderling, & Vallera.1986). Patients exhibiting chimerism harbor both major and minor component DNA. The major component DNA is derived from the donor cells which have repopulated the bone marrow. The minor component DNA remains as surviving host DNA. Simple HLA typing techniques are sufficient to identify major component HLA types but may be inadequate to detect very small quantities of specific minor component DNA (Chalmers, Sproul, Mills, Gibson, and burnett, 1990). The polymerase chain reaction may overcome this obstacle.

Polymerase Chain Reaction

The polymerase chain reaction technique was developed by Mullis in 1983 (Mullis, Faloona, Scharf, Saiki, Horn, & Erlich, 1986). It is a procedure to amplify, in the test tube, a specific gene sequence into millions of copies. The reaction as first described, was catalyzed by the addition of the Klenow fragment of Escherichia coli DNA polymerase I enzyme. This enzyme had an optimum activity at 37^{o}C. The low temperature for maximum enzyme activity limited this enzyme's use, since the higher temperatures necessary to separate double stranded DNA into single stranded DNA inactivated the enzyme. This made it necessary to add fresh enzyme during every cycle of the reaction (Saiki, Scharf, Faloona, Mullis, Horn, Erlich, & Arnheim, 1985).

The introduction of a thermostable DNA polymerase enzyme removed the need to add enzyme at each cycle. The thermostable enzyme is produced by the bacterium Thermus aquaticus and the enzyme is known as Taq polymerase (Saiki, Gelfand, Stoffel, Scharf, Higuchi, Horn, Mullis, & Erlich, 1988).

The polymerase chain reaction has been utilized to increase the detection sensitivity for chimerism following allogeneic bone marrow transplantation. Male cells were detected among female cells following a female bone marrow donation to her brother. Some male cells survived the pretransplantation eradication treatment and were detected by amplifying a portion of the Y chromosome. The Y chromosome is not found in females, so only the male DNA was amplified (Lawler, McCann, Conneally, & Humphries, 1989).

PCR can be used to distinguish between genetically different tissues (Jeffreys, Wilson, Neumann, & Keyte, 1988). The technique amplifies hypervariable regions of DNA. The hypervariable regions examined thus far include the following: repeating nucleotide sequences known as variable number of tandem repeats (VNTR), portions of the Y chromosome, and HLA-DQ alpha segments.

VNTR is a nucleotide base sequence within the DNA that is repeated many times (Jeffreys, Wilson & Thein, 1985). Each individual is distinguished by the number of repeating sequences. The number of repeats determines the size of the amplified VNTR, and the size determines the migration distance on gel electrophoresis. Thus a difference in migration

distance between two samples of amplified VNTR may distinguish between two genetically different individuals.

Taq Polymerase

Strain YT1 of Thermus aquaticus is a thermophilic microorganism capable of growth between 70° and 75°C. It was isolated from a hot spring in Yellowstone National Park 22 years ago (Brock & Freeze, 1969). Its thermophilic characteristics permit its DNA polymerase a relatively high optimum enzyme activity temperature. The substitution of this thermostable DNA polymerase enzyme for the Escherichia coli DNA polymerase I led to extensive PCR use in molecular biology research (Saiki, Gelfand, Stoffel, Scharf, Higuchi, Horn, Mullis & Erlich, 1988).

Streptavidin-Biotin Detection System

The PCR technique provides amplified DNA fragments. Detection and differentiation of these amplified products is complex. Biologic products, including amplified DNA molecules, may be present in very small quantities. Low concentrations present unique detection and assay problems. Additional detection labels may be necessary to enhance detection. Fluorescent, radioactive, and enzyme molecules are examples of common labels. These labels are bound to a probe such as an antibody or a complimentary DNA strand. The specificity of the detection system depends on the specific probe, but the specific test visualization depends on the label.

Detection sensitivity varies with the choice of labels. There are two methods for label testing. The first is a direct method. The second is

an indirect method.

Avidin-biotin and streptavidin-biotin technology is an indirect technique to enhance labeling properties without interfering with the basic reaction. The two molecules, streptavidin and biotin have binding affinities for each other. This affinity facilitates the formation of a bridge between two desired locations (Green, 1975).

Biotin is a water soluble vitamin that is easily coupled to proteins, DNA and other organic molecules. Molecules bound to biotin are called biotinylated. The molecular weight is only 244 daltons (Green, 1975). The small size allows biotin to be conjugated to many sites without interfering with the activity of the larger molecule (Bayer, Skutelshy & Wilchek, 1990).

The AmpliType system used to detect specific DQ alpha genes (DNA) utilizes biotin, covalently bonded to an amplified segment of the DQ alpha gene. The attached biotin does not interfere with the activity of the amplified segment (Cetus Corporation, 1990).

Avidin is a glycoprotein antibiotic-like factor found in the whites of some avian eggs. The molecular weight is 68,000 daltons (Kogel & Tonnis, 1936). Avidin has an extremely strong binding affinity for biotin (Green, 1975). Chaiet reported avidin-like activity in various Streptomyces species. This substance is known as streptavidin (Chaiet, Miller, Tansig, & Wolf, 1963).

Streptavidin is independently coupled to an enzyme, horseradish peroxidase. Biotinylated HLA-DQ alpha DNA binds to specific base pairs of

a complimentary DNA probe. Streptavidin-horseradish peroxidase will form a bridge between the biotin on the DNA and the substrate for the enzyme, horseradish peroxidase. If there is no biotinylated DNA complementary to the DNA probe, there will be no enzyme available for the substrate. The last step is to introduce the substrate for the enzyme. The substrate changes color if the enzyme has been bound. Binding only occurs if the streptavidin coupled to the horseradish-peroxidase binds biotin. Biotin-streptavidin binding occurs only if there was annealing of complimentary DNA strands. Color formation indicates the enzyme acted on it's substrate; thus, annealing occurred and amplified DNA was detected (Cetus Corporation, 1990).

Restriction Endonucleases

Bacteria contain endonucleases that cut double-stranded DNA at specific recognition sites. Restriction endonucleases are isolated from bacteria and used as research tools to cut DNA at specific nucleotide sites (Maniatis, Fritsch & Sambrook, 1982).

Polyacrylamide Gel

Gel electrophoresis through polyacrylamide gel, is a method for resolving mixtures of DNA molecules (Peacock & Dingman, 1968). DNA is placed on the polyacrylamine gel and is caused to migrate through the gel. At a neutral pH, DNA is negatively charged and migrates from the negative cathode to the positive anode of an electrophoresis instrument. The mobility is dependent on fragment size. Smaller DNA fragments migrate faster than larger ones. Polyacrylamide gels can be used for separation of

DNA fragments between 6 base pairs (20% polyacrylamide) and 1000 base pairs (3% polyacrylamide) (Sealy & Southern, 1982).

The polyacrylamide gel results from the polymerization of acrylamide monomers into linear chains and the linking of these chains with N,N'-methylenebisacrylamide (bis). The concentration of acrylamide to bis determines the pore size of the resulting gel and hence its sieving effect on DNA of different sizes (Maniatis, Jeffery & Van de Sande, 1975).

A gradiant polyacrylamide gel is one in which the concentration of polyacrylamide varies throughout the gel (Sealy & Southern, 1982). One of the NOVEX polyacrylamide gels is of this type. It has a concentration of polyacrylamide that varies from 4% to 20% (NOVEX Corporation, 1990).

CHAPTER III

Materials and Methods

Evaluation Specimens

Test kit evaluation and supplemental kit analysis utilized DNA specimens from three patients of known HLA-DQ alpha types. The specimens were obtained from patients previously tested for their HLA-DR (DR) type using restriction fragment length polymorphism (RFLP) methodology. The DR type correlates with the HLA-DQ (DQ) type. Thus, by knowing the DR type, the DQ type is known (Saiki, et.al., 1986). The DR typing was performed in Dr. George Moxley's laboratory in the Department of Medicine, Division of Rheumatology, Allergy, and Immunology at the Medical College of Virginia, Richmond, Virginia.

Each DNA specimen was homozygous for a single DQ alpha type. The three known patient DQ alpha types were 1.1, 1.2, and 3.0. These three representative types were chosen because of their base sequences.

The 1.1 DQ alpha type and the 1.2 DQ alpha type differ from each other by only one nucleotide base. The 1.1 DQ alpha type differs from the 3.0 DQ alpha type by 15 nucleotide bases and the 1.2 DQ alpha type differs

by 16 nucleotide bases. The nucleotide base sequences are shown in Figure 1.

DNA Extraction

Ten milliliters (mL) of whole blood were collected in EDTA tubes. To isolate the white blood cells (WBCs), 10 mL of whole blood were mixed gently with 40 mL of red blood cell (RBC) lysing solution for one minute. The lysing solution consisted of a mixture of 0.32 M sucrose, 0.01 M Tris at pH 7.5, 0.005 M $MgCl_2$, and 1% Triton X-100. The specimen was centrifuged for 40 minutes at 2750 revolutions per minute (RPM). The supernatant was discarded; the WBC button was resuspended with 5 mL of RBC lysing solution. This was recentrifuged for 10 minutes at 2750 RPM followed by decanting the supernate. The WBC button was incubated for 20 minutes in a solution of 40 µL of mercaptoethanol and 5 mL of guanidine isothiocyanate. Five mL of isopropanol were added to precipitate the DNA from the dissolved WBC button. The DNA was washed with 10 mL of 70% ethanol and recentrifuged for 10 minutes at 2750 RPM. The supernate was decanted; the DNA resuspended in 0.4 mL of TE (10 mM Tris with 1 mM EDTA, pH of 8.0).

The concentration of DNA in µg/mL was determined with a Hoefer Scientific Instruments DNA Fluorometer employing the Hoechst dye technique. Specimens were stored at 4°C.

DQ Alpha Test Kit Amplification Reagents

PCR amplification reagents consisted of 1.6 mL of 8 mM $MgCl_2$ in

1.1 DQ Alpha

TTT GAT GGA GAT GAG GAG TTC TAC GTG GAC CTG GAG AGG AAG GAG ACT GCC TGG CGG TGG CCT GAG TTC AGC AAA T TT GGA GGT T TT GAC CCG CAG GG

1.2 DQ Alpha

TTT GAT GGA GAT GAG CAG TTC TAC GTG GAC CTG GAG AGG AAG GAG ACT GCC TGG CGG TGG CCT GAG TTC AGC AAA TTT GGA GGT TTT GAC CCG CAG GG

3.0 DQ Alpha

TTT GAT GGA GAC GAG GAG TTC TAT GTG GAC CTG GAG AGG AAG GAG ACT GTC TGG CAG TTG CCT CTG TTC CGC AGA TTT AGA AGA TTT GAC CCG CAA TT

FIGURE 1. Nucleotide base sequences of DQ alpha types 1.1, 1.2, and 3.0. Nucleotide base differences of the 1.2 and 3.0 types, as compared to the 1.1 type, are underlined. Sequences are listed 5' to 3'.

distilled water, 5 mL of mineral oil and 100 μL of 100 ng/mL human genomic DNA of known DQ alpha type.

The kit included 24 tubes, each containing 50 μL of a PCR mixture. The mixture consisted of two HLA-DQ alpha sequence specific biotinylated primers, Amplitaq DNA polymerase, deoxyadenosine 5'-triphosphate (dATP), deoxyguanosine 5'-triphosphate (dGTP), deoxycytidine 5'-triphosphate (dCTP), and deoxythymidine 5'-triphosphate (dTTP). All amplification reagents were stored at 4°C.

DQ Alpha Test Kit Amplification Procedure

One test tube was used for each sample DNA or positive control. Fifty μL of 8mM $MgCl_2$ solution were added to each tube containing 50 μL of the PCR mixture. The mixture was overlayed with two drops of sterile mineral oil. One μg of sample DNA was pipetted under the mineral oil into the PCR mixture. Two ng of the genomic DNA were added to the positive control tube. The tubes were sealed and inserted into the thermal cycler for amplification. The thermal cycler was programmed for 36 cycles. Each cycle was set to denature at 94°C for 1.5 minutes, anneal at 60°C for 1 minute, and extend at 72°C for 1 minute. The last cycle had an additional extension time of 8 minutes.

After amplification, the reaction mixture underlying the mineral oil was removed and placed in a fresh capillary tube. The tube was capped, labeled, and stored at 4°C. Amplification products were later detected

with gel electrophoresis and were typed with the DQ alpha test kit probe strips or other techniques.

DQ Alpha Test Kit Typing Reagents

Typing reagents consisted of 0.8 mL of horseradish peroxidase-streptavidin conjugate, 30 mg of 3,3',5,5' tetramethylbenzidine (TMB) reconstituted with 15 mL of 95% ethanol, three DNA typing trays, each with eight wells containing a DNA probe strip, and 24 HLA-DQ alpha DNA probe strips. Each probe strip contained nine sequence-specific DNA probes for six HLA-DQ alpha alleles. These typing reagents were stored at 4°C.

Citrate buffer was made by dissolving 18.4 g of trisodium citrate dihydrate ($Na_3C_6H_5O_7$-$2H_2O$) in 800 mL of distilled water. The pH was adjusted to 5.0 by the addition of citric acid monohydrate. The final volume was adjusted to 1 liter using distilled water. Twenty percent (w/v) sodium dodecyl sulfate (SDS) solution was prepared by dissolving 200 g of SDS in distilled water and then adjusting the volume to 1 liter with distilled water. Twenty-X sodium chloride-sodium phosphate-ethylenediaminetetraacetic acid (SSPE) buffer was prepared by dissolving 7.4 g of EDTA-$2H_2O$ in 800 mL distilled water and adjusting the pH to 6.0 with 10 N NaOH. Two hundred ten grams of NaCl and 27.6 g of NaH_2PO_4-H_2O were added together with 10 mL of NaOH to adjust to pH 7.4. The final volume was adjusted to 1 liter with distilled water. The wash solution was prepared by mixing 250 mL of the 20X SSPE, 10 mL of the 20% SDS, and adjusting with distilled water to 2 liters. The hybridization solution

was prepared by mixing 250 mL of 20X SSPE, 25 mL of 20% SDS, and adjusting with distilled water to 1 liter. All solutions were stored at room temperature. Prior to use, the hybridization and wash solutions were warmed to 37°C.

The color development solution was prepared within 10 minutes of use by mixing 10 mL of citrate buffer, 10 µL of 3% hydrogen peroxide, and 0.5 mL of TMB, for each probe strip to be tested.

DQ Alpha Typing

Amplified DNA was heated to 95°C for 5 minutes to denature the double-stranded DNA into single-strands. Thirty-five µL of the DNA were added to the sample well of the typing tray containing a probe strip in a mixture of 3 mL of freshly prepared hybridization/enzyme conjugate solution. The solution was prepared by mixing 3.3 mL hybridization solution at 37°C and 27 µL horseradish peroxidase-streptavidin conjugate for each strip used. The tray was incubated at 55°C for 20 minutes in a water bath at 50 rpm after which the hybridization solution was aspirated from the sample wells. Ten mL of wash solution were added to each sample well. The tray was rocked gently for 3 seconds and the wash solution was aspirated from the sample wells. Ten mL of wash solution were added to each sample well and the tray incubated at 55°C for 20 minutes in a water bath at 50 rpm after which the wash solution was aspirated from the sample wells. An additional 10 mL of wash solution

were added to the sample wells, and the tray incubated at room temperature for 5 minutes on an orbital shaker at 50 rpm. The solution was aspirated from the sample wells. Ten mL of citrate buffer were added to the sample wells; the tray was incubated at room temperature for 5 minutes on an orbital shaker at 50 rpm. The citrate buffer was aspirated. Ten mL of color development solution were added and the tray incubated at room temperature for 60 minutes on an orbital shaker at 50 rpm protected from light. The solution was aspirated. Ten mL of distilled water were added and the tray incubated at room temperature for 5 minutes on an orbital shaker at 50 rpm. The distilled water was aspirated from the sample wells. This step was repeated two additional times, then the probe strip was tested for color development.

Color development time was extended from 30 minutes (Cetus recommended) to 60 minutes to enhance sensitivity of detection and to test specificity. Color development less intense than the control probe spot was interpreted as a positive result because control experiments showed that this reactivity did not occur in the absence of the indicated DQ alpha type.

Allele-Specific Amplification

The goal of allele-specific amplification was to increase or amplify small quantities of the specific DQ alpha type (the minor component DNA) in the presence of a relatively larger quantity of another DQ alpha type (the major component DNA). Two approaches were attempted. The first approach used a PCR reaction mixture made within the laboratory. The

minor component DNA was amplified with allele-specific primers. The second approach used the Cetus kit tubes containing the test kit PCR reaction mixture and blocked allele-specific oligonucleotides which were designed to prevent amplification of major component DNA.

In the first approach four allele-specific primers were used. The allele-specific primers had nucleotide sequences that bind or anneal to a known specific nucleotide base sequence on the DQ alpha DNA as seen in Figure 6. This allowed amplification of a portion of the DQ alpha gene. The number of bases incorporated in the amplification products was determined by the annealing site of the primers. The nucleotide base sequences of the primers were specifically selected to anneal the primers at specific locations on the DNA which were characteristic of the three DQ alpha types tested.

The 1.0 DQ alpha allele-specific primer was designed to amplify 1.1 or 1.2 DQ alpha DNA in the presence or absence of 3.0 DQ alpha DNA.

The 1.1 DQ alpha allele-specific primer amplified 1.1 DQ alpha DNA in the presence or absence of 1.2 DQ alpha DNA. The 1.2 DQ alpha allele-specific primer amplified 1.2 DQ alpha DNA in the presence or absence of 1.1 DQ alpha DNA. Thus, a specific DQ alpha DNA might be amplified with an allele-specific primer even in the presence of DNA of a different DQ alpha type.

Two primers were used in each allele-specific amplification test tube. One primer was the allele-specific primer used to amplify the minor component DNA; the other was a biotinylated primer designated RS 152.

The RS 152 primer was biotinylated at the 5' end, and its base sequence allowed it to be used with any of the four allele-specific primers mentioned above; therefore, it is not an allele-specific primer. The biotinylated primer, or RS 152, was identical to one of the two primers found in the Cetus test kit. This primer was used with each of the four allele-specific primers mentioned above when performing amplification. The resulting amplification products were biotinylated to facilitate typing by the test kit probe strips.

The PCR mixture volume was 100 μl. The PCR mixture contained 10 μL each, of an allele-specific primer and of the RS 152 primer, 16 mL of 25 mM $MgCl_2$, 2.5 μl each of the four deoxynucleotide 5'-triphosphates (dNTPs), 10 μl of 10X Tris-KCl PCR buffer, and 5 μl of a 1:10 dilution of AmpliTaq DNA polymerase. One μg of major component DNA and 1:500 dilution (2 ng) of minor component DNA was added to the reaction mixture. The final volume of the reaction mixture was adjusted to 100 μl with sterile, distilled water stored at room temperature. The mixture was overlayed with two drops of sterile mineral oil stored at room temperature. The test tube was sealed and placed in the thermal cycler for amplification.

The thermal cycler was programmed for 40 cycles. Each cycle was set to denature at 94°C for 1.5 minutes, anneal at 44°C for 1 minute, and extend at 72°C for 1 minute. The last cycle had an additional extension time of 8 minutes.

After amplification, the reaction mixture underlying the mineral oil was removed and placed in a fresh capillary tube. The tube was capped, labeled, and stored at 4°C. Amplification products were detected later with gel electrophoresis and were typed with the DQ alpha test kit probe strips. This procedure identified the DQ alpha type of the amplified products and thus of the minor component DNA.

$MgCl_2$, dNTPs, PCR buffer and the polymerase were stored at minus 20°C prior to use. The primers were stored at 4°C.

The second approach used a blocked allele-specific oligonucleotide to prevent amplification of the major component DNA. Four blocked allele-specific oligonucleotides, each with their 3' hydroxyl group replaced with an amine group, were prepared. They were identical to the allele-specific primers used in the first approach, but had a blocking amine molecule in place of their 3' hydroxyl group. This configuration does not permit DNA polymerase to add nucleotides to the oligonucleotide. Although the oligonucleotide anneals to the DNA, there is no extension of nucleotides; thus, no new DNA forms.

The blocked 1.0 DQ alpha allele-specific oligonucleotide was used to prevent amplification of 1.1 or 1.2 DQ alpha DNA in the presence of 3.0 DQ alpha DNA. The blocked 1.1 DQ alpha allele-specific oligonucleotide was used to prevent amplification of 1.1 DQ alpha DNA in the presence of 1.2 DQ alpha DNA. The blocked 1.2 DQ alpha allele-specific oligonucleotide was used to prevent amplification of 1.2 DQ alpha DNA in the presence of 1.1 DQ

alpha DNA.

This latter approach used the Cetus kit test tubes, containing the Cetus PCR reaction mixture. To this mixture, was added 50 μL of 8 mM $MgCl_2$, 10 μL of a blocked allele-specific oligonucleotide, two drops of mineral oil, and 1 μg of major component DNA. The test tube was sealed and placed in the thermal cycler for amplification. The thermal cycler was programmed for 38 cycles. Each cycle was set to denature at 94°C for 1.5 minutes, anneal at 44°C for 1 minute, and extend at 72°C for 1 minute. The last cycle had an extension time of 8 minutes.

After amplification, the reaction mixture underlying the mineral oil was removed and placed in a fresh capillary tube. The tube was capped, labeled, and stored at 4°C. Amplification products were detected with gel electrophoresis.

All allele-specific primers and blocked allele-specific oligonucleotides were purified by high pressure liquid chromatography (HPLC) and dried. Prior to use, they were reconstituted with sterile, distilled water to a final concentration of 100 μg/mL and stored at 4°C.

NOVEX Xcell Mini-Cell Electrophoresis Instrument

PCR products were electrophoresed for two reasons. One was to verify that DNA was amplified. This was done prior to probe strip typing to ensure the costly strips were only used when amplified products existed. The second reason for electrophoresis was to confirm restriction endonuclease activity. The gel can detect and visualize bands of

electrophoresed PCR product that had undergone restriction endonuclease action.

Twenty microliters (μL) of PCR product mixed with 4 μL of sample buffer were loaded into sample wells. Stock sample buffer consisted of a mixture of 6.0 mL of 5X TBE, 3.2 mL of glycerol, 0.3 mL of 1% bromophenol blue, 0.3 mL of 1% xylene cyanol, and 0.2 mL of distilled water.

The instrument was filled with 700 mL of running buffer. Stock running buffer consisted of a mixture of 108 g of Tris base, 55 g of boric acid, 5.8 g of EDTA, and 2 L of distilled water.

PCR products were electrophoresed at 125 constant volts for approximately 1 hour. Starting current ranged from 6-12 mA and ending current ranged from 3-6 mA. Four to 20% polyacrylamide gradient gels of 1.0 mm thickness and configured with 10 sample wells were obtained from NOVEX corporation, Encinitas, California.

A biotinylated gel marker (Research Genetics, Huntsville, Alabama) was included with each gel electrophoresed. The gel marker contained biotinylated double-stranded DNA bands of 1000, 700, 500, 400, 300, 200, 100, and 50 base pairs. The biotinylated gel marker was used to visualize the bands after Southern blot transfer from the polyacrylamide gel onto a nylon membrane.

A Southern transfer was performed after the gel was stained for 10 minutes in distilled water containing 0.5 μg/mL ethidium bromide followed by a 30 minute distilled water wash to visualize bands using an

ultraviolet (UV) transluminator. Gels were then photographed.

NOVEX Southern Transfer to Nylon Membrane

DNA bands in the electrophoresed gel were transfered to a nylon membrane by a Southern blotting technique, the Southern transfer. This transfer placed DNA bands from the gel to the exact location on the nylon membrane.

NOVEX transfer membranes were pre-cut 8.3 X 7.3 cm; assembled sandwich style with two pieces of Whatman 3 mm filter paper. The membrane was a microporous nylon membrane modified with strongly basic charged groups.

Transfer buffer was a mixture of 108 g Tris base, 55 g boric acid, 5.8 g EDTA, and 2 L distilled water. Exactly 150 mL of transfer buffer were added to the inside transfer chamber. This was insulated by 700 mL of water placed in the outside chamber. Southern transfer was performed for 1 hour at constant 20 volts; the current ranged from 250-300 mA.

The purpose of the transfer was to provide a target for a detection system, the Enzygraphic Web. After Southern transfer to the nylon membrane was completed, the membrane was prepared for application of the web. This preparation involved several steps. Initially, the nylon membrane underwent blocking at room temperature while gently rocking for 2 minutes with 50 mL of solution consisting of 5% SDS and 1% Tween 20, both in tris buffered saline (TBS). Blocking prevented nonspecific binding between the membrane and the horseradish peroxidase-streptavidin conjugate. A 1 mg/mL stock solution of horseradish

peroxidase-streptavidin conjugate was obtained from Calbiochem. Fifty mililiters of a 1:1000 dilution of stock solution was allowed to bind biotinylated DNA on the nylon membrane. The solution was gently rocked for 10 minutes at room temperature.

The blocking process was repeated at room temperature while gently rocking for 10 minutes. This was followed with three washes for 10 minutes, each at room temperature in a solution of 0.05% Tween 20 in TBS. Two final wash steps were each performed with 50 mL of TBS for 10 minutes at room temperature while gently rocking.

DNA Detection With Enzygraphic Web

The Enzygraphic Web is a support polymer coated with a patented detection system for peroxidase enzyme activity. Amplified DNA was electrophoresed in gel; the DNA bands in gel were Southern transferred to a nylon membrane. After transfer, horseradish peroxidase-streptavidin conjugate was bound to amplified DNA via DNA bound biotin. Application of the Enzygraphic Web to the membrane allowed the horseradish peroxidase to undergo an enzymatic reaction creating a blue color within seconds on the web. The frosted surface of the Enzygraphic Web was layed on top of the membrane with sufficient hand pressure to create an even contact. Target DNA appeared on the Enzygraphic Web within seconds.

Restriction Endonucleases

Mnl I was obtained from Biolabs, Beverly, Massachusetts and Hae III was obtained from Bethesda Research Laboratories, Gaithersburg, Maryland. Mnl I was expected to cut amplified DNA of the 1.1 DQ alpha type

and not 1.2 or 3.0 DQ alpha types. Hae III cuts amplified DNA of both the 1.1 DQ alpha and the 1.2 DQ alpha types. Hae III does not cut amplified DNA of the 3.0 DQ alpha type.

The purpose for using the restriction endonucleases was to selectively cut amplified DNA at specific base sequence sites prior to examination by gel electrophoresis. Since the DNA from the various DQ alpha types have different base sequences, one DQ alpha type can be cut while another DQ alpha type remains uncut. When examined by gel electrophoresis, the cut DQ alpha DNA should reveal bands, while the uncut DNA should be seen as one single band of 242 base pairs.

This might allow identification of the major component DNA and the minor component DNA. Eighteen microliters of the amplified DNA were added to 2 μL of the appropriate endonuclease buffer and 1 μL of the appropriate endonuclease. The mixture was allowed to incubate for 1 hour at 37°C before being electrophoresed on the NOVEX polyacrylamide gel. The gel then underwent ethidium bromide staining to allow a photograph. The gel was then southern transferred to a nylon membrane.

The nylon membrane was overlayed with the Enzygraphic Web. The Enzygraphic Web was expected to increase the detection sensitivity of transferred bands.

CHAPTER IV

Results

AmpliType HLA-DQ Alpha Test Kit

Known, homozygous, DQ alpha genes were amplified to verify the AmpliType HLA-DQ Alpha test kit specificity. The amplified specimens were tested for hybridization to known complimentary DNA. The complimentary DNA was bound to probe strips provided by the Cetus AmpliType HLA-DQ Alpha test kit. Hybridization was detected on the probe strips by a streptavidin bound, horseradish peroxidase - TMB enzymatic color development (a blue dot). This is illustrated in Figure 2. The kit suggests a 30 minute color development incubation and recording, but a second reading at 60 minutes was also performed. Both readings had identical results.

The 1.1 DQ alpha DNA reacted positively with probes complementary for 1, 1.1, control (C) and all but 1.3 (Figure 3). The results indicate the amplified DNA to be DQ alpha type 1.1. The 1.2 DQ alpha DNA reacted positively with probes located at positions 1, 1.2/1.3/4, C, and all but 1.3. These results indicate the amplified 1.2 DQ alpha DNA typed correctly as 1.2. The 3.0 DQ alpha DNA reacted positively with probes at positions 3.0,

The Generation of a Dot Blot Using Cetus Immobilized Allele-Specific Oligonucleotide Probes

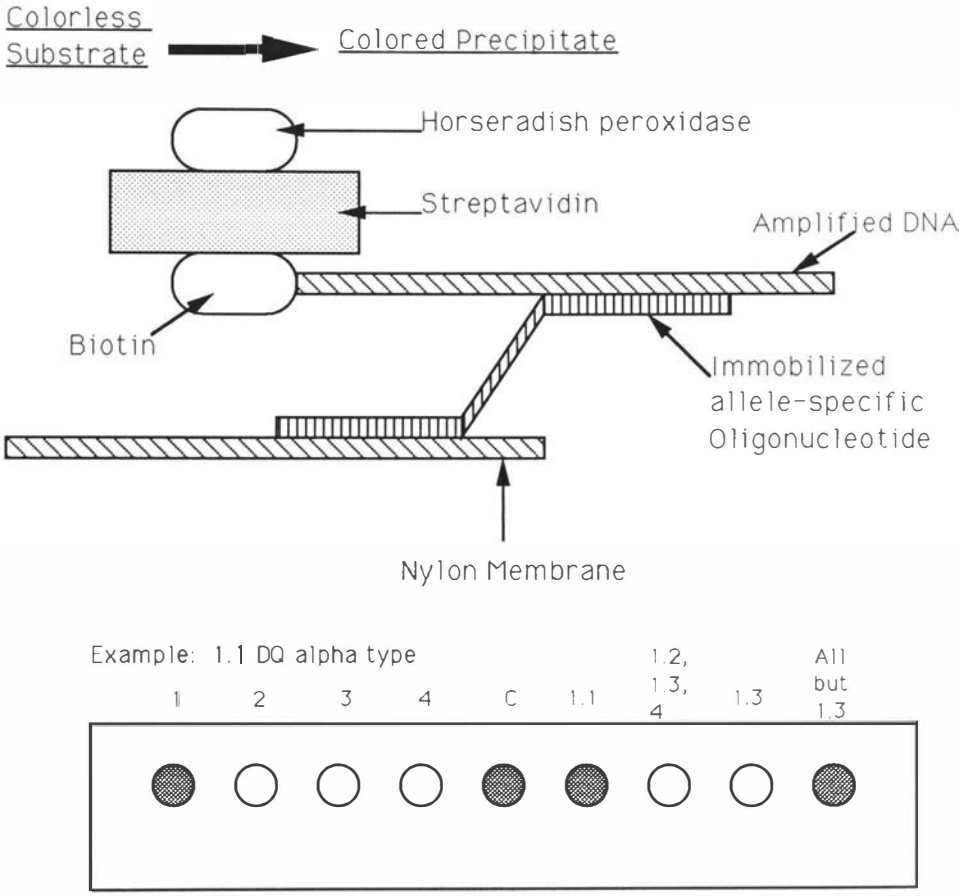

FIGURE 2. Top of figure shows amplified DNA annealed to allele-specific oligonucleotide. The biotinylated DNA binds streptavidin which is conjugated to horseradish peroxidase. The peroxidase causes colorless TMB to form a colored precipitate. Bottom of figure shows color formation of DQ alpha type 1.1.

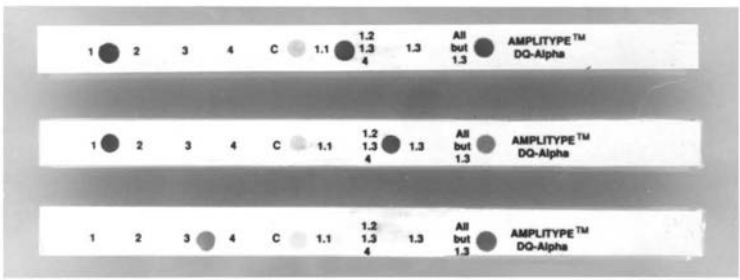

FIGURE 3. Three AmpliType DQ alpha probe strips used to type amplified DNA for DQ alpha type. The top probe strip was reacted with DNA from an individual homozygous for 1.1 DQ alpha. The middle strip was reacted with DNA from an individual homozygous for 1.2 DQ alpha. The bottom strip was reacted with DNA from an individual homozygous for the DQ alpha type of 3.0.

C and all but 1.3. These results indicate correct typing for DQ alpha type 3.0.

Amplified DNA was diluted and typed for DQ alpha using the probe strips. Color development time was extended from 30 minutes to 60 minutes to enhance sensitivity of detection. Single specimens of amplified DNA accurately typed at a dilution of 1:1000 for each of the three DQ alpha types used. The specimens were not detectable at a dilution of 1:2000 for each of the three DQ alpha types.

Amplified 1.1 DQ alpha DNA was mixed with 1:500, 1:1000 and 1:2000 dilutions of amplified 3.0 DQ alpha DNA. The three mixtures were typed with probe strips. Color developed at the 1.1 DQ alpha DNA region in the three mixtures. The 3.0 DQ alpha DNA showed color development at the 1:500 dilution. The 1:1000 and 1:2000 dilutions did not show color development for the 3.0 DQ alpha DNA.

Amplified 1.1 DQ alpha DNA was also tested undiluted with 1:500, 1:1000, and 1:2000 dilutions of amplified 1.2 DQ alpha DNA. The three mixtures were typed with known probe strips. Color developed at the 1.1 DQ alpha DNA region in the three mixtures. The 1.2 DQ alpha DNA showed color development at the 1:500 dilution. The 1:1000 and 1:2000 dilutions did not show color development for the 1.2 DQ alpha DNA.

DQ alpha typing was also performed on DNA samples mixed prior to amplification and also resulted in probe strip color development. Color development time was extended from 30 minutes to 60 minutes to enhance sensitivity of detection. The minor component DNA showed less intense

color development than the control probe spot. In the normal application of the test kit, this color pattern is interpreted as an inconclusive result. In adapting the kit to detect chimerism, this color pattern was interpreted as a positive result because this weaker reaction only occurred if the minor component was present.

Unamplified and undiluted 1.1 DQ alpha DNA was mixed with 1:500, 1:1000, and 1:2000 dilutions of unamplified 1.2 DQ alpha DNA. The mixtures were amplified, and the resulting amplification products were typed with known probe strips. Color developed at the 1.1 DQ alpha DNA region in all mixtures. In addition, the 1.2 DQ alpha DNA showed color development at the 1:500 dilution. The 1:1000 and 1:2000 dilutions did not show color development for the 1.2 DQ alpha type.

Unamplified and undiluted 1.2 DQ alpha DNA was mixed with 1:500, 1:1000, and 1:2000 dilutions of unamplified 1.1 DQ alpha DNA. The mixtures were amplified, and the resulting amplification products were typed using probe strips. Color developed at the 1.2 DQ alpha DNA region in all mixtures. In addition, the 1.1 DQ alpha DNA showed color development at the 1:500 dilution. The 1:1000 and 1:2000 dilutions did not show color development for the 1.1 DQ alpha type.

Unamplified and undiluted 1.1 DQ alpha DNA was also mixed with 1:500, 1:1000, and 1:2000 dilutions of unamplified 3.0 DQ alpha DNA. The two mixtures were amplified and the resulting PCR products were typed using probe strips. Color developed at the 1.1 DQ alpha DNA region in all mixtures. As seen in Figure 4, the 3.0 DQ alpha DNA showed color

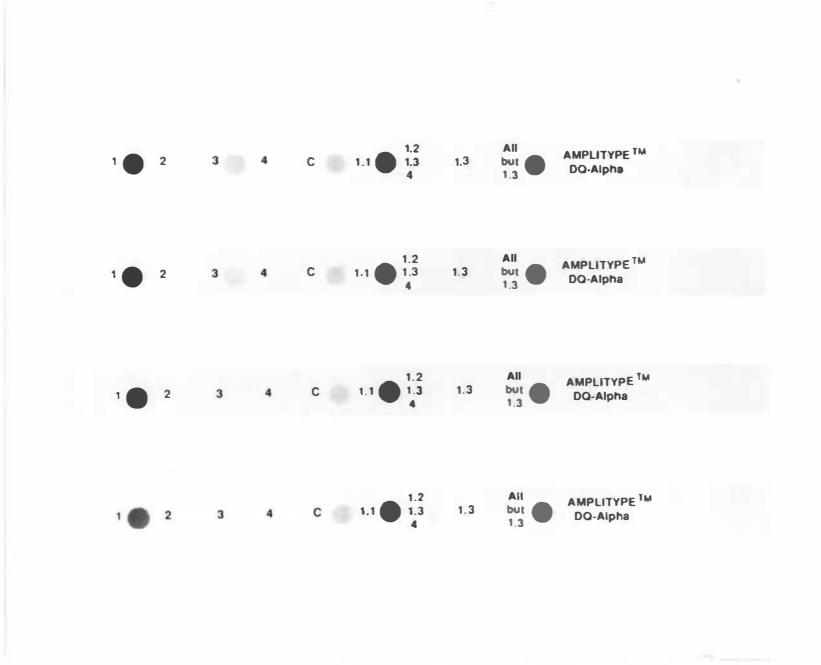

FIGURE 4. Four AmpliType DQ alpha probe strips used to type amplified DNA for DQ alpha type. From top to bottom: first strip shows preamplified 1.1 DQ alpha DNA mixed undiluted with 1:500 dilution of preamplified 3.0 DQ alpha DNA. Second strip shows preamplified 1.1 DQ alpha DNA mixed undiluted with 1:1000 dilution of preamplified 3.0 DQ alpha DNA. Third and fourth strips are undiluted 1.1 DQ alpha DNA from two separate aliquots of stored DNA specimen run as controls. All were amplified prior to strip testing.

development at the 1:500 and 1:1000 dilutions. The 1:2000 dilution did not show color development for the 3.0 DQ alpha type.

Allele-Specific Amplification

Allele-specific amplification was an added alternative technique to amplify minor component DNA in the presense of a major component DNA of a different DQ alpha type.

Allele-specific amplification for the three different DQ alpha types was evaluated. The nucleotide base sequence of the allele-specific primers is seen in Figure 5. Color development time was extended from 30 minutes to 60 minutes to enhance sensitivity of detection. Single specimens of amplified DNA accurately typed when undiluted and at a dilution of 1:1000 for each of the three DQ alpha types used. As seen in Figure 6, amplification begins at the allele-specific primer and continues 5' to 3'. Probe areas located distal (3') to the allele-specific primer annealing location tested positive. The 1.1 allele-specific primer amplified the 1.1 DQ alpha DNA only. The PCR products reacted positively with probes located at positions 1.1, C, all but 1.3, and 1.0. The 1.2 allele-specific primer amplified the 1.2 DQ alpha DNA only. The PCR products reacted positively with probes located at positions 1.2/1.3/4.0, C, all but 1.3, and 1.0. The 3.0 allele-specific primer amplified the 3.0 DQ alpha DNA only. The PCR products reacted positively with the probe located at position 3.0. The 1.0 allele-specific primer amplified the 1.1 DQ alpha DNA. The PCR products reacted positively with the probe located at position 1.0. The 1.0 allele-specific primer amplified the 1.2 DQ alpha

DQ 1.0: TCA GCA AAT TTG GAG

DQ 1.1: TTG ATG GAG ATG AGG

DQ 1.2: TTG ATG GAG ATG AGC

DQ 3.0: TGT TCC GCA GAT TTA

FIGURE 5. Nucleotide base sequences of the allele-specific primers. Sequences are listed 5' to 3'.

1.1 DQ Alpha

TTT GAT GGA GAT GAG GAG TTC TAC GTG GAC CTG GAG AGG AAG GAG ACT GCC TGG CGG TGG CCT GAG TTC AGC AAA TTT GGA GGT TTT GAC CCG CAG GG
TT GAT GGA GAT GAG G
TC AGC AAA TTT GGA G

1.1 primer

1.0 primer

1.2 DQ Alpha

TTT GA T GGA GAT GAG CAG TTC TAC GTG GAC CTG GAG AGG AAG GAG ACT GCC TGG CGG TGG CCT GAG TTC AGC AAA TT T GGA GGT TTT GAC CCG CAG GG
TT GAT GGA GAT GAG C
TC AGC AAA TTT GGA G

1.2 primer

1.0 primer

3.0 DQ Alpha

TTT GAT GGA GAC GAG GAG TTC TAT GTG GAC CTG GAG AGG AAG GAG ACT GTC TGG CAG TTG CCT CTG TTC CGC AGA TTT AGA AGA TTT GAC CCG CAA TT
TG TTC CGC AGA TTT A

3.0 primer

FIGURE 6. Nucleotide base sequences of DQ alpha types 1.1, 1.2, and 3.0 showing annealing sites of the 4 allele-specific primers. The primers anneal to the DNA strand complementary to the sequences listed above. Sequences are listed 5' to 3'.

DNA. The PCR products reacted positively with the probe located at position 1.0.

Amplified 1.1 DQ alpha DNA was mixed undiluted with 1:500 and 1:1000 dilutions of amplified 1.2 DQ alpha DNA. The two mixtures were typed using the probe strips. Color developed at the 1.1 DQ alpha DNA region in the two mixtures. The 1.2 DQ alpha DNA showed color development at the 1:500 dilution. The 1:1000 dilution did not show color development for the 1.2 DQ alpha DNA.

Amplified 3.0 DQ alpha DNA was mixed undiluted with 1:500 and 1:1000 dilutions of amplified 1.1 DQ alpha DNA. The two mixtures were typed using the probe strips. Color developed at the 3.0 DQ alpha DNA in the two mixtures. The 1.1 DQ alpha DNA showed color development at the 1:500 dilution. The 1:1000 dilution did not show color development for the 1.1 DQ alpha DNA.

DQ alpha typing was also performed on DNA samples mixed prior to amplification and also resulted in probe strip color development. Color development time was extended from 30 minutes to 60 minutes to enhance sensitivity of detection. The minor component DNA showed less intense color development than the control probe spot. In the normal application of the test kit, this color pattern is interpreted as an inconclusive result. In adapting the kit to detect chimerism, this color pattern is interpreted as a positive result because this weaker reaction only occurred if the minor component was present.

The mixtures were amplified using allele-specific primers for the

minor component DNA DQ alpha types and the amplification products evaluated. All gave appropriate bands on gel electrophoresis at the 1:500 dilutions. In addition, the 1:500 dilutions for the minor component DNA DQ alpha types all gave positive reactions when tested with the probe strips indicating appropriate specificity and sensitivity down to a 1:500 dilution.

Unamplified and undiluted 1.1 DQ alpha DNA was mixed with 1:500 and 1:1000 dilutions of unamplified 1.2 DQ alpha DNA. In addition, unamplified and undiluted 3.0 DQ alpha DNA was mixed with 1:500 and 1:1000 dilutions of unamplified 1.1 DQ alpha DNA. The mixtures were amplified, and the resulting amplification products were typed using probe strips. The results showed color development for the major component DQ alpha type in both mixtures. The minor component DQ alpha DNA showed color development at the 1:500 dilution in both mixtures. The 1:1000 dilution did not show color development for the minor component DQ alpha type in both mixtures.

Allele-specific oligonucleotides blocked at their 3' hydroxy group were used for the three different DQ alpha types. These were used with the Cetus kit tubes in an attempt to prevent Cetus primers from amplifying the major component. This was not the case as the amplification products from this experiment gave appropriate bands of 242 base pairs when electrophoresed on NOVEX polyacrylamide gel.

These altered primers were not needed to block major component DNA amplification in the reaction cocktail which also used allele-specific primers for the minor component. The allele-specific primers for the

minor component DNA DQ alpha type amplified the minor component, even in the presense of major component DNA of a different DQ alpha type. The amplification products typed appropriately with probe strips at a 1:500 dilution.

Restriction Endonucleases

The reason for using restriction endonuclease enzymes was to selectively cut amplified DNA at specific base sequence sites prior to examination by polyacrylamide gel electrophoresis. Cut DNA was electrophoresed, Southern blotted to a nylon membrane, then an Enzygraphic Web containing a color development system was applied to the nylon membrane to increase detection sensitivity.

The restriction endonuclease Mnl I consistently cut nonspecifically and could not be used to differentiate between amplification products of DQ alpha types 1.1 and 1.2. However, the enzyme Hae III did cut the amplification products where expected. Hae III cut amplified DNA of DQ alpha types 1.1 and 1.2 into three bands each. Hae III appropriately did not cut amplified DNA of DQ alpha type 3.0. Figure 7 shows expected restriction sites for Mnl I and Hae III.

The NOVEX polyacrylamide gel electrophoreses system was used to evaluate the amplification products. As seen in Figure 8, a 1:2000 dilution of amplified DNA of the 3.0 DQ alpha type gave an appropriate band on ultraviolet (UV) illumination at 254 nm. The biotinylated marker gave an appropriate band at a 1:1000 dilution. The same gel (Figure 8) underwent a Southern transfer to a nylon membrane to be reacted with the Enzygraphic

1.1 DQ Alpha

TTT GAT GGA GAT GAG GAG TTC TAC GTG GAC CTG GAG AGG AAG GAG ACT GCC TGG CGG TGG CCT GAG TTC AGC AAA TTT GGA GGT TTT GAC CCG CAG GG

Mnl I Hae III

1.2 DQ Alpha

TTT GAT GGA GAT GAG CAG TTC TAC GTG GAC CTG GAG AGG AAG GAG ACT GCC TGG CGG TGG CCT GAG TTC AGC AAA TTT GGA GGT TTT GAC CCG CAG GG

Hae III

3.0 DQ Alpha

TTT GAT GGA GAC GAG GAG TTC TAT GTG GAC CTG GAG AGG AAG GAG ACT GTC TGG CAG TTG CCT CTG TTC CGC AGA TTT AGA AGA TTT GAC CCG CAA TT

FIGURE 7. Nucleotide base sequences of DQ alpha types 1.1, 1.2, and 3.0. Site of restriction endonuclease action is underlined. Sequences are listed 5' to 3'.

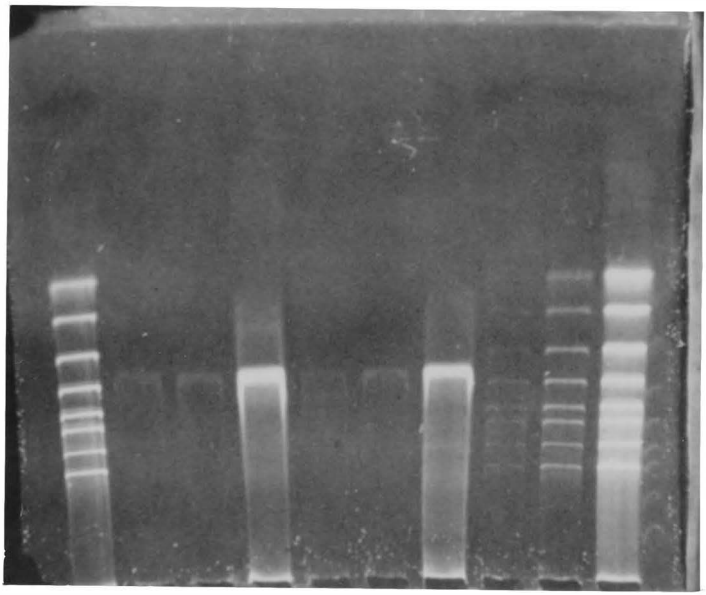

FIGURE 8. Polyacrylamide gradient gel electrophoresis with ethidium bromide staining. DQ alpha amplification products were treated with restriction endonucleases prior to electrophoresis. From left to right; lane 1 is 1:10 biotinylated gel marker, lane 2 is 1:2000 dilution of 3.0 DQ alpha DNA, lane 3 is 1:1000 dilution of 3.0 DQ alpha DNA, lane 4 is 1:10 dilution of 3.0 DQ alpha DNA, lane 5 is 1:2000 dilution of 3.0 DQ alpha DNA, lane 6 is 1:1000 dilution of 3.0 DQ alpha DNA, lane 7 is 1:10 dilution of 3.0 DQ alpha DNA, lane 8 is 1:1000 dilution of the biotinylated marker, lane 9 is 1:100 dilution of the biotinylated marker, lane 10 is undiluted biotinylated marker.

Web. Application of the Enzygraphic Web is illustrated in Figure 9. The results of this application of the Enzygraphic Web are seen in Figure 10. A faint band is seen at a 1:2000 dilution of amplified DNA. The biotinylated marker gave an appropriate band at a 1:100 dilution.

Hae III was reacted with a mixture containing amplified and undiluted 1.1 DQ alpha DNA mixed with 1:1000 dilution of amplified 3.0 DQ alpha DNA. Hae III was also reacted with a mixture containing amplified and undiluted 1.2 DQ alpha DNA mixed with 1:1000 dilution of amplified 3.0 DQ alpha DNA. Hae III was also reacted with two additional mixtures. These were mixed before amplification and contained 1:1000 3.0 DQ alpha DNA mixed with undiluted 1.1 DQ alpha DNA in one tube and undiluted 1.2 DQ alpha DNA in another tube. All mixtures were electrophoresed on polyacrylamide gel and revealed appropriate bands for the 1.1 and 1.2 DQ alpha types. The 1:1000 dilutions of the 3.0 DQ alpha type were not visible on the gel. A Southern transfer was performed to evaluate sensitivity of the Enzygraphic Web. The Enzygraphic Web showed appropriate bands for the 1.1 and 1.2 DQ alpha types. The 1:1000 dilutions of the 3.0 DQ alpha type were not visible on the Enzygraphic Web.

Detection of Peroxidase Linked DNA with Enzygraphic Web

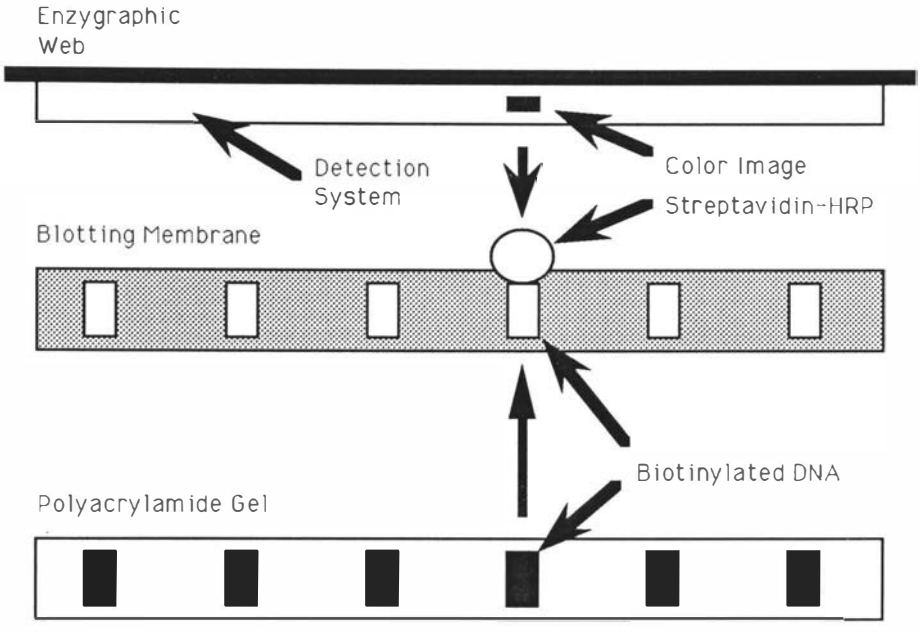

FIGURE 9. Enzygraphic Web is used to detect streptavidin-horseradish peroxidase linked DNA. The Enzygraphic Web is placed in direct contact with the blot under ordinary room light. The color image is formed on the Web within seconds.

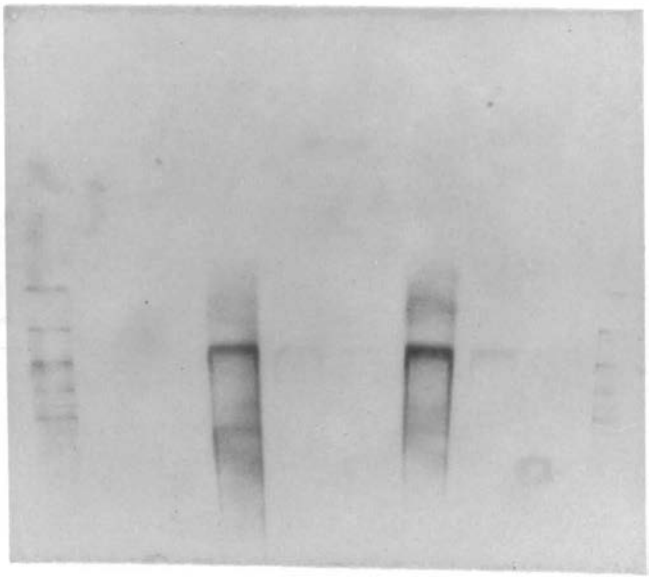

FIGURE 10. Enzygraphic Web applied to NOVEX gel seen in figure 7. Gel underwent Southern Blotting to a nylon membrane with application of Enzygraphic Web to the nylon membrane. From right to left; lane 1 is 1:10 biotinylated gel marker, lane 2 is 1:2000 dilution of 3.0 DQ alpha DNA, lane 3 is 1:1000 dilution of 3.0 DQ alpha DNA, lane 4 is 1:10 dilution of 3.0 DQ alpha DNA, lane 5 is 1:2000 dilution of 3.0 DQ alpha DNA, lane 6 is 1:1000 dilution of 3.0 DQ alpha DNA, lane 7 is 1:10 dilution of 3.0 DQ alpha DNA, lane 8 is 1:1000 dilution of the biotinylated marker, lane 9 is 1:100 dilution of the biotinylated marker, lane 10 is undiluted biotinylated marker.

CHAPTER V

Discussion

Chimerism may be associated with graft-versus-host disease in allogeneic bone marrow transplantation patients (Borgaonkar, et.al., 1974). Graft-versus-host disease is a life-threatening complication in these patients (Hansen, et.al., 1981).

Most recently, studies by Lawler, et al., showed that the polymerase chain reaction increased the detection sensitivity for chimerism in allogeneic bone marrow transplantation patients using primers for the Y chromosome (Lawler, et.al., 1989). The polymerase chain reaction has been used to amplify the hypervariable region of the DQ alpha gene. DQ alpha gene polymorphism may be selectively tested to distinguish between DNA from different individuals (Gyllensten, et.al., 1988). To date, amplification of the DQ alpha gene has not been used to detect chimerism.

This was a study of detection systems for DQ alpha HLA polymorphism. This polymorphism was exploited to demonstrate simulated chimerism. The polymorphic region of the DQ alpha gene was amplified by the polymerase chain reaction and the amplification products characterized. Techniques included probe strip typing, allele-specific

amplification, polyacrylamide gel electrophoresis, restriction enzymes, and Southern transfer combined with the Enzygraphic Web.

The AmpliType HLA-DQ Alpha Test Kit performed very well. In simulated chimeric mixtures, the minor component DNA was specifically detected when samples were diluted 1:500. This represents a detection sensitivity of 0.2%. This is much better than 10% detection sensitivity in non-PCR techniques (Lawler, et.al., 1989).

In a simulated chimeric mixture diluted 1:500, the one base difference between the 1.1 and 1.2 DQ alpha types was detected by the probe strips. When the 3.0 DQ alpha type was mixed as the minor component in a simulated chimeric mixture with undiluted 1.1 DQ alpha type, the detection sensitivity for the 3.0 DQ alpha type increased to 1:1000 dilution, a detection level of 0.1%.

One reason for the difference in the detection sensitivities could be heteroduplex formations (Wenger & Nielsen, 1991), or reannealing between the 1.1 and 1.2 DQ alpha types. The 1.1 and 1.2 DQ alpha types could be reannealing to each other because they are similar; there is only a 1 base difference. In a mixture of 1.1 and 1.2 DQ alpha, the minor component could be reannealing to the major component leaving less minor component to hybridize to the probe strips. This leads to a weaker probe strip color signal and may explain why the minor components of 1.1 and 1.2 DQ alpha were negative at 1:1000 while the 3.0 DQ alpha was positive when mixed with the major component of 1.1 DQ alpha.

Erlich suggests a potential problem amplifying small amounts of

DNA in the presence of larger quantities of a different DQ alpha type. This is known as the plateau effect (Erlich, 1989). Allele-specific amplification was performed to compensate for the plateau effect. The minor component DNA was selectively and efficiently amplified in the presence of a major component DNA of different non-amplified DQ alpha type (Erlich, 1989).

The plateau effect did not affect minor component DQ alpha detection. The allele-specific primers were able to specifically amplify the minor component DNA. The major component DNA did not amplify, and thus, did not compete with the minor component DNA for Taq polymerase. Thus, the allele-specific primers compensated for the plateau effect.

The blocked allele-specific primers should have prevented amplification of the major component DNA. They did not prevent amplification of the major component. At the extension temperature of 72^{o}C, the non-extendable blocking primer may not anneal to its complementary site.

The restriction endonuclease Hae III has been used by Maeda to differentiate 1.1 or 1.2 from 3.0 DQ alpha type using undiluted and amplified DQ alpha DNA (Maeda, Murayama, Ishii, Uryu, Ota, Tsuji & Inoko 1989). Maeda indicated that Mnl I can be used to differentiate between 1.1 and 1.2 DQ alpha types. Mnl I cut nonspecifically and could not be applied.

Hae III worked well and was used to detect minor component DQ alpha types 1.1 or 1.2 in the presence of major component DQ alpha type 3.0. Background interference prevented detection of minor component

bands.

Limitations

Restriction endonuclease testing was limited. Background interference prevented detection of minor component DNA in mixtures simulating chimerism. This occurred in both polyacrylamide gels stained with ethidium bromide and on Southern transferred membranes reacted with the Enzygraphic Web.

Recommendations

The AmpliType HLA-DQ Alpha test kit successfully amplified minor component DNA in a simulated chimeric mixture. The allele-specific primers performed similarly and thus did not offer any advantage over selective amplification of the minor component DNA.

The AmpliType HLA-DQ Alpha test kit offers the best method of detecting chimerism, as demonstrated with simulated chimeric mixtures. However, the possible effect of heteroduplex formation on the ability of minor component DQ alpha DNA to hybridize to probe strips requires further investigation. This investigation could lead to an increased detection sensitivity to 1:1000 for the minor component DNA in the presence of major component DNA of any DQ alpha type.

Restriction endonuclease testing requires further investigation. The current dilution for detection of minor component DNA would not permit restriction endonucleases to be a viable alternative to the AmpliType HLA-DQ Alpha test kit. Perhaps alternative non-radioactive or radioactive techniques would permit detection of these components.

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